# New sensitive assay for phosphatidylserine decarboxylase based on the detection of $CO_2$ from nonradiolabeled phosphatidylserine

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**Summary** A new rapid assay for phosphatidylserine decarboxylase, which is sensitive in the nanomolar range, is described. Synthesis of radiolabeled phosphatidylserine for use as a substrate is not required, since the assay, unlike previous ones, is based on the detection of $CO_2$ liberated from unlabeled phosphatidylserine. The assay employs a gas chromatographic procedure for the analysis of methane formed by catalytic conversion of the $CO_2$ produced as a product of the enzymatic reaction.

**Supplementary key words** phosphatidylserine · carbon dioxide detection · *Tetrahymena pyriformis*

Phosphatidylserine decarboxylase is usually assayed by the production of $^{14}CO_2$ from chemically synthesized dipalmitoyl glycerophosphoryl[1-$^{14}$C]serine (1–4). An assay based on the detection of $^{14}$C-labeled phosphatidylethanolamine formed as a result of the action of the decarboxylase on enzymatically prepared $^{14}$C-labeled phosphatidylserine has also been employed (5). We wish to report here the development of a new assay for phosphatidylserine decarboxylase which is both rapid and sensitive in the nanomolar range (as is the previously used assay [1–4]) but which offers the advantage of not requiring the involved synthesis of radiolabeled phospholipid for use as a substrate. This assay is based on the gas chromatographic detection of unlabeled $CO_2$ and should also be useful in the assay of other decarboxylases and for the determination of $CO_2$ where greater sensitivity is required than that provided by manometric techniques.

In order to determine the amount of $CO_2$ present in the assay sample by gas chromatographic methods, the $CO_2$ must first be converted to methane for detection by a flame ionization detector. This is necessary because $CO_2$ itself does not burn and thermal conductivity detectors do not provide the sensitivity desired. The conversion was achieved by modification of a standard flame ionization gas chromatograph so that the carrier gas is hydrogen instead of nitrogen and after the sample is passed through a column for the separation of $CO_2$ from other gases, the sample is passed over a nickel nitrate catalyst in a heater block at 325°C, which converts the $CO_2$ to methane in the presence of hydrogen. The conversion of carbon monoxide to methane for gas chromatographic detection has been previously reported (6).

## Materials and methods

A particulate preparation of phosphatidylserine decarboxylase was prepared from a culture of *Tetrahymena pyriformis* W (strain 10542) obtained from the American Type Culture Collection. The procedure of Dennis and Kennedy (3) was followed, except that cell growth was carried out at room temperature on 40 l of growth medium contained in a glass carboy. The lyophilized powder obtained from a typical preparation contained about 3.5 g protein, as determined by the method of Lowry et al. (7). Samples of this protein were resuspended in buffer (50 mM imidazole–HCl, pH 7.4) containing 12 mM EDTA, centrifuged (48,000 g, 0°C, 30 min), washed once with buffer and once with distilled water, and relyophilized; the EDTA-washed protein was used in the assays.

Bovine brain phosphatidylserine was obtained from Schwarz/Mann (lot X 1138) and purified by DEAE-cellulose chromatography using an adaptation of the procedure of Papahadjopoulos and Miller (8) for separating brain phospholipids; this procedure was first described by Rouser et al. (9). In a typical preparation, 1.5 g of the commercial phosphatidylserine was applied to a column of Whatman "microgranular" DE 32 cellulose (2.5 × 35.5

Abbreviations: Phosphatidylserine, 1,2-diacryl-*sn*-glycero-3-phospho-L-serine; dipalmitoyl glycerophosphorylserine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl-L-serine; NMR, nuclear magnetic resonance.

cm) in chloroform–methanol 7:3, which had been prepared according to the manufacturer's specifications. The column was eluted with chloroform–methanol 7:3 (550 ml) and then with methanol (350 ml), and the effluents were discarded. Then glacial acetic acid (1000 ml) was used to elute the phosphatidylserine, and the solvent was removed by lyophilization. To ensure that all of the resulting phosphatidylserine was in the acid form, the phospholipid was washed with HCl according to the procedure of Rathbone, Magee, and Thompson (10). The resulting free acid form of phosphatidylserine was taken up in benzene and lyophilized in tared vessels. The resulting dry phosphatidylserine was stored under argon at −20°C. The purified phosphatidylserine gave a single spot on thin-layer chromatography. The thin-layer chromatography was carried out on precoated layers of silica gel G on glass plates (Brinkmann Instruments) and developed in two dimensions: (i) chloroform–methanol–ammonium hydroxide 13:7:1 and (ii) chloroform–acetone–methanol–acetic acid–water 5:2:1:1:0.5, according to the procedure of Rouser, Siakotos, and Fleischer (11). The phospholipids were visualized after treatment of the plates with the phosphorus reagent of Dittmer and Lester (12) and then by treatment with 90% methanol. Sodium and potassium content of the purified phosphatidylserine was shown to be negligible as determined by atomic absorption spectroscopy in isopentyl acetate on a Varian Techtron model AA-4 atomic absorption spectrometer according to a modification of the method of Montford and Cribbs (13). A phosphorus assay based on a modification of the method of Chen, Toribara, and Warner (14) suggested a molecular weight for the phosphatidylserine of about 790. Nuclear magnetic resonance spectra taken on a Varian HR 220 NMR spectrometer were consistent with pure phosphatidylserine.

Saturated phosphatidylserine was prepared by hydrogenation of the purified phosphatidylserine. In a typical preparation, 0.45 mmoles of phosphatidylserine was hydrogenated in $CHCl_3$–ETOH 1:1 with a Brown[2] hydrogenator (Delmar Scientific Laboratories) and $PtO_2$ catalyst (J. Bishop & Co.). Hydrogenation was allowed to proceed for 19 hr, after which 1.09 mmoles of $H_2$ was taken up; the apparatus was calibrated with cyclohexene. The uptake of $H_2$ corresponds to an average of 2.4 double bonds/molecule of phospholipid. The resulting phospholipid was washed according to the procedure of Rathbone et al. (10) and lyophilized from benzene. The resulting white material gave a single spot on thin-layer chromatography as described above, and the 220 MHz NMR spectrum of the phospholipid was consistent with full saturation.

Triton X-100 was obtained from Rohm and Haas Co. and was used without further purification. All other chemicals were of reagent grade.

*Assay of phosphatidylserine decarboxylase.* A Varian Aerograph gas chromatograph containing a flame ionization detector, model 2100, and a Varian recorder equipped with a disc integrator, model 20, were modified so that the carrier gas line to one injector port was switched with the hydrogen line to the corresponding burner; thus the carrier gas became hydrogen and the burner was fed with nitrogen and compressed air (and hydrogen from the column). The injector port was connected to copper tubing in order to carry the gas sample outside of the column oven and into a glass column (6 ft × ¼ inch OD, 2 mm ID) at room temperature, packed with Pora-pak type Q, mesh 80–100 (Waters Associates). The column was then connected via copper tubing and Swagelok fittings to a "reactor tube" consisting of a piece of column tubing (13.5 cm long) packed with 7 cm of 5% nickel nitrate catalyst embedded on firebrick support, 40–60 mesh (Varian Aerograph), stoppered with glass wool, and connected via copper tubing back into the chromatograph's detector flame head. The reactor tube was itself contained in a ¼ inch hole in an aluminum heating block (2 × 3 × 3 inches) containing two Deenergize heaters (Western Control Co.) located perpendicular to the reaction tube and a Vulcan thermostat (Western Control Co.). The temperature of this heating block was monitored by a thermocouple lead attached to the temperature indicator of the gas chromatograph and was maintained at 325°C. Under typical conditions, the retention time for $CO_2$ was about 1 min; successive samples could be injected every 2–3 min.

Under "standard conditions," the assay system consisted of 50 mM imidazole–HCl, pH 7.0, 1.5 mM phosphatidylserine as the free acid, 4 mM Triton X-100, and 1.25 mg of protein in a total volume of 0.5 ml. Incubations were conducted for 20 min at 22°C. The reaction vessel consisted of a 2-dm sample vial (Arthur H. Thomas Co.) and a plastic Reacti-Vial cap containing a center hole (Pierce Chemical Co.) and fitted with a 13-mm silicone rubber septum (Applied Science Laboratories). These vessels are gas tight and the total capacity is 5.0 ml. In order to conduct the assay, a brass manifold containing Luor Lok fittings and disposable Yale syringe needles (22 gauge, 1.5 inches long) were employed. Reaction vessels were arranged so that their caps were held in place above them by the syringe needles protruding through their septa. The vessels were flushed with argon and then the reactants were pipetted into the vessels under mild argon flow. Reaction was initiated by the addition of protein (homogenized dispersion of the EDTA-washed lyophilized powder). The reaction vessels were capped, and the contents were thoroughly mixed in a Vortex mixer. The vessels were then placed in a thermostated water bath–shaker. Reaction was stopped by the addition of 1.0 ml of 1.0 N $H_2SO_4$ by syringe, and then the contents of the

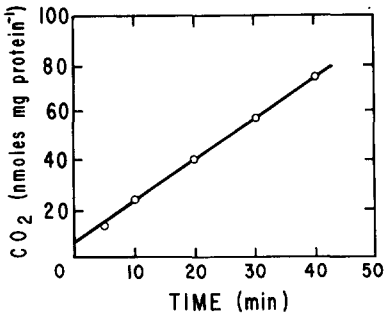


**Fig. 1.** Time course of action of phosphatidylserine decarboxylase towards phosphatidylserine. Standard assay conditions were employed, except for the variation with time.

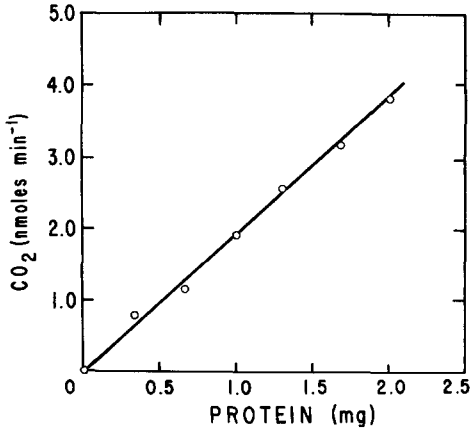


**Fig. 2.** Protein dependence of phosphatidylserine decarboxylase activity towards phosphatidylserine. Standard assay conditions were employed, except for the variation of added protein.

reaction vessels were gently mixed and allowed to equilibrate for 20 min at room temperature. Controls were obtained by immediately adding the $H_2SO_4$ after capping the vessels. The addition of acid stops the enzymatic reaction and, by lowering the pH of the solution, also liberates the $CO_2$ to the air space in the vessel.

In order to determine the amount of $CO_2$ liberated, a calibrated gas-tight Hamilton syringe (Hamilton Co.), fitted with a Traylor slug and flushed with nitrogen before use, was employed to obtain a 1.00-ml aliquot of the air space in each vessel. The amount of $CO_2$ produced in the reaction vessel was calculated from the $CO_2$ content of the 1.00-ml aliquot which was determined gas chromatographically by comparison with a standard sample of $CO_2$ prepared from bone-dry $CO_2$ (Matheson Coleman & Bell) diluted in argon (Liquid Carbonics) and analyzed at the same time. The chromatographic system could easily detect as little as 1 nmole of $CO_2$ in a 1.00-ml gas sample; the standard error of analyses of several 21-nmole samples of $CO_2$, prepared as described above, was $\pm 1\%$. A standard sample was included in every experiment, but its value did not change significantly between experiments. Although the reaction vessels were completely flushed with argon before use to remove $CO_2$ from the air space in the vessels, the controls include a large amount of $CO_2$ that is dissolved in the reactants used. The total amount of dissolved $CO_2$ is consistently about 25 nmoles, and the amount does not vary greatly between experiments. Thus, controls were included in every experiment and are subtracted from the experimental points. The results reported here are the averages of duplicate assays; the error in the duplicate assays was less than $\pm 5\%$.

Where noted, phosphatidylserine decarboxylase was also assayed by the release of $^{14}CO_2$ from dipalmitoyl glycerophosphoryl[1-$^{14}$C]serine according to a previously described procedure (3). Dipalmitoyl glycerophosphoryl-[1-$^{14}$C]serine was the gift of Dr. E. P. Kennedy.

## Results and Discussion

Using the assay procedure described herein, the activity of phosphatidylserine decarboxylase towards natural phosphatidylserine as substrate is about 2 nmoles min$^{-1}$ mg protein$^{-1}$. The activity is linear with time for at least 40 min, as shown in Fig. 1, although if the best-fit line is extrapolated to zero time, it does not pass through the origin. The high intercept is consistently obtained; its magnitude appears to be related to the amount of active protein added in the presence of phosphatidylserine, and it does not appear to be due to any impurity in the assay mix, phosphatidylserine, or protein. Thus, this assay

TABLE 1. Phosphatidylserine decarboxylase activity towards various substrates using both the gas chromatographic and the radioactive assays

| Assay[a] | Substrate | Triton X-100 | $CO_2$ |
|---|---|---|---|
| | | | *nmoles min$^{-1}$ mg protein$^{-1}$* |
| Radioactive | Dipalmitoyl glycerophosphoryl[1-$^{14}$C]serine | + | 0.42 |
| | | − | 0.16 |
| Gas chromatographic | | + | 0.60 |
| | | − | 0.28 |
| | Phosphatidylserine | + | 1.24 |
| | | − | 0.73 |
| | Saturated phosphatidylserine | + | 0.35 |
| | | − | 0.13 |

[a] The assay system consisted of 50 mM imidazole–HCl, pH 7.0, 0.2 mM substrate, 4 mM Triton X-100 where indicated, 5 mM EDTA, and 2.5 mg of protein in a total volume of 0.5 ml. Incubations were conducted for 20 min at 35°C.

may be used effectively to determine relative activities by comparing $CO_2$ production at 20 min (standard assay conditions) even though the precise cause of the high intercept is not understood clearly at this time. Because of the need to control $CO_2$ backgrounds in this assay, it was deemed advantageous to use the detailed assay procedure described here. However, it is also possible to preequilibrate the assay tubes and add the enzyme by syringe at zero time. This requires extra care to prevent the addition of air along with the enzyme, but results comparable to those reported here can be obtained by this modified procedure. The enzyme activity is also linear with added protein, as shown in Fig. 2.

As shown in Table 1, the gas chromatographic assay described here and the previously used radioactive assay (3) give similar results when the same dipalmitoyl glycerophosphoryl[1-$^{14}$C]serine is used as substrate in both assays, although the results do not agree precisely. The lack of perfect agreement is not surprising, however, since the two assays are not read against the same standard samples, are conducted in different types of vessels, and involve different techniques, and the gas chromatographic assay involves a large background control compared to the radioactive assay. Table 1 also shows that natural phosphatidylserine shows greater activity than either the saturated phosphatidylserine or the synthetic dipalmitoyl glycerophosphorylserine, and the synthetic glycerophosphorylserine shows somewhat greater activity than the saturated material. All three substrates show an activation by Triton X-100 under the experimental conditions employed, as had been previously reported with the synthetic substrate using the radioactive assay (3). The differences in the activity of phosphatidylserine decarboxylase towards the natural and the saturated substrates may reflect actual specificity of the enzyme or differences in the physical states of the different substrates (15). We have used this new assay to determine the activity of phosphatidylserine decarboxylase towards various unlabeled substrates and to assess the role of Triton X-100 in this system more precisely; we will report these results separately. [1]

This investigation was supported by a National Science Foundation grant GB-19056 and a National Institutes of Health grant NS-09326. T. G. Warner is a PHS Predoctoral Trainee on grant GM 1045 from the National Institute of General Medical Sciences.

*Manuscript received 29 January 1973; accepted 29 May 1973.*

[1] Warner, T. G., and E. A. Dennis. Manuscript in preparation.

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